Proc. Natl. Acad. Sci. USA
Vol. 76, No. 9, pp. 4360–4364, September 1979
Biochemistry

Cloning of the structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12

(transmembrane protein/radioimmunoassay/minicells)

ULF HENNING, HANS-DIETER ROYER†, RON M. TEATHER‡, INGRID HINDENNACH, AND CORNELIS P. HOLLENBERG

Max-Planck-Institut für Biologie, 7400 Tübingen, West Germany

Communicated by Wolfgang Beermann, June 18, 1979

ABSTRACT The gene (*ompA*) for the major outer membrane protein II* from *Escherichia coli* K-12 has been cloned on a 5-megadalton *Eco*RI fragment by using phage λ as vector. The gene is expressed during the lytic cycle of the recombinant phage and the insoluble membrane-bound protein was detected in phage plaques with a simple radioimmunoassay. Transfer of the *Eco*RI fragment into plasmid pSC101 and expression in a host lacking protein II* led to overproduction of protein II* and decreased production of two other major outer membrane proteins. Expression of the plasmid pSC101-*ompA*+ in minicells derived from an *ompA* minicell-producing strain led to synthesis, at high rates, of this protein and massive accumulation of a second cell envelope protein most likely representing the biosynthetic precursor of protein II*.

Polypeptide II* (1) is one of the few so-called major or abundant proteins of the *Escherichia coli* cell envelope (for other such proteins and other nomenclatures see ref. 2). The protein (M_r ≈33,000), present at about 10^5 copies per cell, spans the outer membrane of the cell (3) and can serve as a receptor for phages K3 and TuII* (4, 5). There is evidence for several physiological functions of the protein (4, 6–9); however, none of these is well defined so far.

The protein is synthesized in precursor form (10), presumably possessing an extended NH_2-terminal signal sequence (11) as has been demonstrated for the outer membrane lipoprotein (12). Except for the finding that outer membrane proteins are inserted into the outer membrane during synthesis (13), nothing is known concerning the mechanism of membrane incorporation, including an answer to the intriguing question of why such proteins are not found in the plasma membrane. Also, nothing is known about the regulation of synthesis of protein II* and of a number of other such proteins. It would thus be desirable to study the synthesis of the protein *in vitro* and for this and other obvious reasons we wished to clone the corresponding structural gene *ompA* (14, 15).

Here we describe the construction of a hybrid plasmid carrying this gene. Two main difficulties had to be overcome: methods to select stringently for the wild-type allele were not available, and the gene product is insoluble under conditions that otherwise allow the detection of proteins translated from cloned DNA fragments (16, 17). The methods developed to overcome the problems should be applicable to other such systems, including eukaryotic membrane proteins.

MATERIALS AND METHODS

Strains and Protein Synthesis in Minicells. *E. coli* K-12 strains used were: C 600-SF8 (from S. Falkow; r_K^- m_K^+, *rec* B^-C^-, *lop* II, *lig*+, *gal*Δ, *str, leu, thi, thr*), P400 (16) (from P. Reeves), one of its *ompA* derivatives resistant to phage TuII* and lacking protein II* (18), minicell-producing mutant χ 984 (19) (from R. Curtiss), one of its derivatives lacking the major outer membrane proteins Ia, Ib, and II* (20), KLF6 (21) (from H.-U. Schairer, carrying F′106), and W620 *recA* (15). Cells were grown at 37°C in LB medium (22); the medium was supplemented with tetracycline (10 μg/ml) when strains harbored plasmid pSC101.

Minicells from stationary phase cultures were isolated by three cycles of sucrose gradient centrifugation (23), suspended (2×10^{10} per ml) in minimal salts medium (24) containing 30% glycerol, and stored frozen in liquid nitrogen (25). For protein synthesis, 10^{10} minicells in 0.5 ml of minimal medium containing 0.4% glucose, 25 μl of methionine assay medium (Difco), 250 units of penicillin, and 10 μCi of [^{35}S]methionine [1350 Ci/mmol (1 Ci = 3.7×10^{10} becquerels); Amersham] were incubated for 2 hr at 37°C. Envelopes were obtained by sonication of minicells suspended in water and centrifugation for 30 min at 60,000 × g. They were taken up in 80 μl of 62.5 mM Tris·HCl, pH 6.8/2% Na dodecyl sulfate/10% (vol/vol) glycerol/5% 2-mercaptoethanol/0.001% bromophenol blue and boiled for 3 min. Samples (5–15 μl) were analyzed by polyacrylamide gel electrophoresis on Laemmli-type slab gels (26) as described in ref. 18 and stained with Coomassie brilliant blue.

Construction of λgt and pSC101 Recombinant Molecules. F′-factor DNA (F′106, see *Results*) was isolated according to Sharp *et al.* (27) with some modifications as detailed by Teather *et al.* (28). λgt arms from phage λgt-*araBAD* (29) (from R. W. Davis) were purified from an *Eco*RI digest by preparative agarose gel electrophoresis and subsequent sucrose gradient centrifugation. Ligated purified λgt arms gave <1% of the number of plaques obtained with the ligation product of a complete *Eco*RI digest of λgt-*araBAD*. λgt arm DNA (0.5 μg) was ligated overnight at 10°C with 0.5 μg of *Eco*RI-digested F′106 DNA in 60 μl of 26 mM Tris·HCl, pH 7.5/12 mM NaCl/10 mM $MgCl_2$/1 mM ATP/20 mM dithiothreitol/0.05 unit of T4 DNA ligase.

Calcium-treated *E. coli* SF8 cells were transfected with the ligation mixture (30). The resulting plaques were extracted and the phage pool was further propagated on *E. coli* C600 $r_K^-m_K^+$ to make a high-titer stock of recombinant λ phages. This phage stock contained about 1% of λgt-*araBAD* phages as tested on MacConkey agar (29).

Plasmid pSC101 DNA (31) was isolated from *E. coli* C600 $r_K^-m_K^+$, digested with *Eco*RI, and ligated with *Eco*RI-digested λgt *ompA*+ DNA. *E. coli* P400 *ompA* was transformed ac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

† Present address: Sidney Farber Cancer Institute, Boston, MA 02115.

‡ Present address: Animal Research Institute, K. W. Neatby Bldg., Ottawa, ON, Canada KIA OC6.

cording to the method described by Cohen *et al.* (32) with the following modifications. Cells of a 40-ml culture in L broth (A_{620} = 0.3) were washed once in 10 mM $CaCl_2$ and suspended in 1 ml of 25 mM $CaCl_2$; 100 μl of cells was mixed with 2 μl of ligation mixture (0.05–0.1 μg of DNA) and held for 45 min at 0°C and then for 10 min at 42°C. L broth (2 ml) was added and the cells were grown for 1–2 hr at 37°C before they were plated on L broth plus tetracycline. Minicell-producing strains were similarly transformed with recombinant DNA but the cells were made competent for transformation in 15 mM $CaCl_2$. The plasmid DNA of positive clones was isolated from 2-ml cultures according to the method described by Meagher *et al.* (33).

RESULTS

Construction of λgt-*ompA*+. We could not select stringently for the *ompA*+ allele. Mutants lacking protein II* do not grow, at 37°C, on nutrient broth containing 0.5 mM EDTA and under some other conditions (34); however, in our strains, secondary mutations of unknown nature arise quite frequently, allowing growth of colonies on such selective media in the absence of protein II*. Recently developed, very sensitive, solid-phase radioimmunoassays allow the screening of large numbers of colonies or phage plaques for specific translation products (16, 17). These methods were found to be inapplicable to protein II* because of its complete insolubility under the assay conditions. We therefore developed another radioimmunoassay that allows screening for *ompA*+ colonies (35). In brief, colonies were replica printed (36) onto filter paper, extracted with organic solvents, and exposed to radioiodinated affinity-purified anti-II* immunoglobulin. Subsequent autoradiography allowed the detection of strains that produce very small amounts of the protein.

In earlier experiments we failed to find the *ompA*+ allele in a hybrid plasmid gene bank from Clarke and Carbon (37). This collection of 2000 strains carries random fragments of the *E. coli* chromosome in colicin E1 plasmids. Shortly thereafter, Nishimura *et al.* (38) reported that a number of genes were not represented in this collection; in particular, the structural gene for another major outer membrane protein, the lipoprotein (39), was not found. Attempts to clone the *ompA* gene in plasmid pBR325 (40) present at 20–30 copies per chromosome were also unsuccessful. The F′ prime factor used for these experiments (F′106, see below) carries the *pyrD* gene in addition to *ompA*, and hybrid plasmids pBR325-*pyrD*$^+$ were recovered with the expected frequency. We therefore considered the possibility that too high a gene dosage for such membrane proteins may be lethal to the cell and thus turned to phage λ as cloning vector.

The λgt vector used (29, 41) can be made a viable molecular hybrid by insertion of an *Eco*RI fragment. For lysogenization, however, such hybrids require integration helper phage, and the formation of such double lysogens occurs at a frequency of about 1% (42). It had already been shown (15) that F′106 (21) carries *ompA*, the structural gene for protein II*. If the *ompA* gene does not contain a cleavage site for *Eco*RI, the gene can be expected at frequencies of 0.1–1% in recombinant λgt molecules constructed by ligation of an *Eco*RI digest of F′106 DNA with purified λgt arms. This means that 10^5–10^6 colonies would have to be screened with an assay that allows one person to examine about 5×10^4 colonies per week (35).

To facilitate the screening, we asked the question, is *ompA*+ expressed during the lytic cycle of the hybrid phage? If so, the possibility existed that the protein synthesized this way would also be incorporated into or at least stick to the cell envelope and become detectable in phage plaques with the radiological filter paper assay. A ligated mixture of *Eco*RI digests of F′106 and λgt DNAs was used to transfect a suitable host and to make a high-titer phage stock. The recombinant phage pool was used to infect a mutant lacking protein II*, yielding about 1000 plaques per plate. The plates were replica printed onto filter paper and about 1% of the plaques on the replicas became radioactively labeled upon exposure to radioiodinated anti-II* immunoglobulin. The corresponding phages were purified by three rounds of single plaque isolation. Fig. 1 shows the result of the radioimmunoassay applied to a mixture of such a hybrid phage and λgti (43).

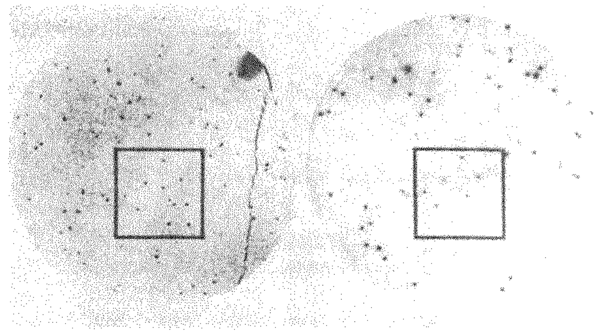

FIG. 1. Phage plaques from a mixture of λgti and λgt-*ompA*+. (*Left*) Plaques on P400 *ompA*. The plate was replica printed onto filter paper which then was treated with radioiodinated anti-protein II* immunoglobulin. (*Right*) Autoradiography (36 hr at −60°C on Kodak X-Omat R film) of the paper. Corresponding areas are in squares.

The isolated phage was used to lysogenize, with the helper phage λgti, an *ompA* mutant lacking protein II* (resistant to phage TuII*). Double lysogens (about 0.5%) were identified with the filter paper radioimmunoassay. They had become fully sensitive to phage TuII* and were found to produce protein II* at wild-type level as judged by visual inspection of stained electrophoretograms (Fig. 2). Expression of the protein by the hybrid phage was not allele-specific—i.e., the protein was produced in 10 *ompA* mutants of independent origin (including 1 nonsense mutant of the amber type). Therefore, it appeared that the desired structural gene with intact control regions had been cloned. The DNA from recombinant phages carrying the *ompA* gene was isolated and an *Eco*RI digest was analyzed by agarose gel electrophoresis. Fig. 3 shows that such phages have integrated a DNA fragment of about 7.5 kilobases.

Plasmid pSC101-*ompA*+. Plasmid pSC101 (present in about six to eight copies per chromosome) confers resistance to tetracycline and possesses one *Eco*RI restriction site (31). A ligated mixture of *Eco*RI digests of this plasmid and λgt-*ompA*+ was used to transform an *ompA* mutant lacking protein II*. About 2% of the colonies resistant to tetracycline were found to be *ompA*+ with the filter paper radioimmunoassay. In contrast to the λgt-*ompA*+ lysogens, such strains were found not only to overproduce protein II* but also to synthesize much decreased amounts of the major outer membrane proteins Ia and Ib (Fig. 2). Although the concentrations of other major outer membrane proteins in P400 *ompA* pSC101-*ompA*+ were not measured quantitatively it was obvious that there was no influence on the concentration of the *tsx* protein but the concentrations of the lipoprotein and the *lamB* protein were also decreased, if only slightly in comparison with polypeptides Ia/Ib (Fig. 2).

Electrophoretic analyses of the plasmid DNA in these strains revealed the presence of an insert of the same size as that found in λgt-*ompA*+. Visual inspection of such electrophoretic patterns revealed a decrease (≈50%) in concentration of

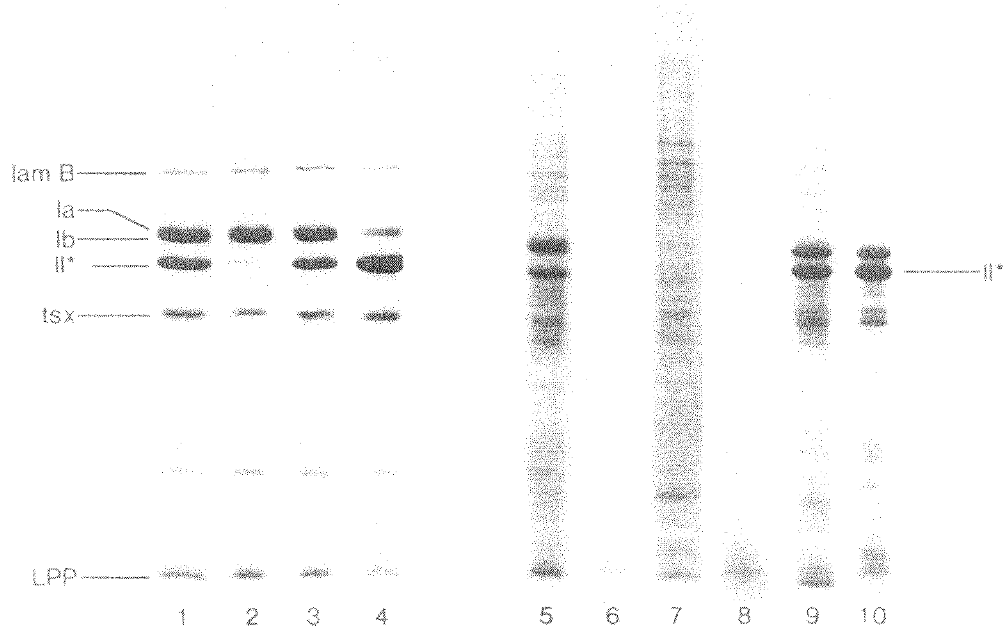

FIG. 2. Electrophoretograms of cell envelopes. Lanes: 1, strain P400 (wild type); 2, P400 *ompA* harboring pSC101 [the same profile was obtained from P400 *ompA* (λgti)]; 3, P400 *ompA* (λgti/λgt-*ompA*+); 4, P400 *ompA* with pSC101-*ompA*+; 5–10, envelopes from minicells upon incubation with [^{35}S]methionine; 5, wild type (stained); 6, autoradiogram of lane 5; 7, *ompA* mutant also lacking proteins Ia/Ib and harboring pSC101 (stained); 8, autoradiogram of lane 7; 9, same mutant as in lane 7 but carrying pSC101-*ompA*+ (stained); 10, autoradiogram of lane 9. Exposure was 48 hr for autoradiograms 6 and 8 and 24 hr for 10. Cells for samples 1–4 were grown in L broth containing maltose to induce phase λ receptor (*lamB* protein). In strain P400 the receptor for phage T6 (*tsx*) is also a major outer membrane protein (44). The weak band in autoradiogram 8 and in a position between *tsx* and II* is probably an ≈26,000-dalton membrane protein specified by pSC101 (45, 46). Note that even in this stationary phase, minicell mRNA for the lipoprotein is still present (autoradiograms 6 and 8).

pSC101-*ompA*+ compared with the same strain transformed with pSC101; this effect has not yet been analyzed further. The plasmid is somewhat unstable. In pSC101-*ompA*+ cultures grown with or without the antibiotic, about 10% of all tetracycline-resistant clones had lost *ompA*+. The plasmid could be stabilized by transfer into strain W620*recA*.

Synthesis of Protein II* in Minicells. To prove that the structural gene for protein II* had been cloned, its synthesis in minicells was studied. An *ompA* minicell-producing strain derived from χ984 was transformed with plasmids pSC101 and pSC101-*ompA*+. Minicells from stationary phase cultures of transformants and of wild-type χ984 were allowed to synthesize protein in the presence of [^{35}S]methionine. Cell envelope proteins were then separated electrophoretically and the ^{35}S-labeled proteins were located by autoradiography. The results in Fig. 2 show that wild-type minicells as well as those lacking protein II* and harboring pSC101 did not incorporate significant amounts of radioactivity into membrane proteins. Minicells from the strain bearing pSC101-*ompA*+ produced *two* heavily labeled polypeptides in large quantities, one in the position of protein II* and another one with a somewhat larger apparent molecular weight (≈35,000). In addition, about 14 polypeptides evident only weakly or not at all as stained bands and with smaller molecular weight than that of protein II* were labeled (Fig. 2). Although, considering the results presented in the preceding sections, the strained and radioactive polypeptide in the position of protein II* is very unlikely to be anything other than this protein, minicell envelopes were also treated with rabbit antiprotein II* serum as described (10, 48). The antiserum precipitated not only the 33,000-dalton protein II* but also the major 35,000-dalton polypeptide (data not shown). The latter is therefore most likely a precursor of protein II* (10). Because the control experiment using wild-type χ984 showed that minicells from stationary phase cells no longer contained the rather stable messenger for protein II*, it is obvious that the structural gene in question has been cloned.

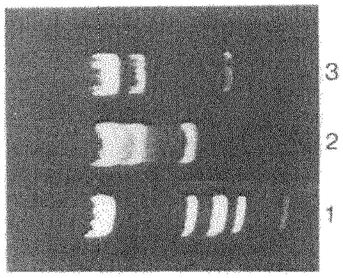

FIG. 3. Agarose gel electrophoresis of *Eco*RI fragments. Lanes: 1, phage λ DNA [molecular weights from left to right (47): 13.7, 4.74, 3.73 plus 3.48 (not separated), 3.02, and 2.13 megadaltons]; 2, λgt-*ompA*+; 3, λgt-*araBAD*.

DISCUSSION

The evidence presented clearly demonstrates that the structural gene for the outer membrane protein II* has been cloned on a 7.5-kilobase *Eco*RI fragment. The nonselective technique used to detect positive recombinant clones should be applicable to other membrane proteins including eukaryotic membrane proteins. In cases in which antibodies are available, an animal cell system could be used—e.g., with SV40 as vector (49). If, as in the case reported here, the gene in question is expressed during the lytic cycle of phage λgt it is no problem to screen 10^5 phage plaques per week.

The cloned *Eco*RI fragment is 7–8 times larger than the gene required to code for protein II*. The interesting phenomena

found upon transfer of this fragment into plasmid pSC101, therefore, may or may not be due to *ompA*+. It is easy and thus very tempting, however, to explain most of them by the presence of that gene. pSC101-*ompA*+ in strain P400 *ompA* leads to an approximately 2-fold increase of protein II* concentration in the outer membrane (compared to wild-type cells), a large effect in view of the fact that in the wild type about 10^5 copies of this protein are present per cell. In such strains the concentrations of two other major outer membrane proteins, polypeptides Ia and Ib, are considerably decreased. This could reflect a competition for common sites where these proteins are inserted into the outer membrane. We have reported earlier that, in a homogenotic merodiploid (*ompA*+/*ompA*+), no gene dosage effect was measurable (15), whereas it has been shown (50) that such an effect does exist for the outer membrane lipoprotein. Double gene dosage for protein II* may not suffice for effective competition with proteins Ia and Ib for translocation, and the lipoprotein may use another site, as appears to be the case for the *lamB* protein (51, 52). It is also conceivable that the existence of the lipoprotein-gene dosage effect is connected with the extraordinary stability of the mRNA for this protein (ref. 53; see also Fig. 2).

In minicells, but not in normal cells, harboring pSC101-*ompA*+, another major envelope protein is detected in addition to protein II*; it exhibits a molecular weight of about 35,000. It is present in large quantity (about 50% that of II*) and it is precipitated by antiserum against protein II*. This fact together with its molecular weight strongly indicates that it represents the precursor of the protein(10). Continued rapid synthesis of protein II* in the absence of cell envelope growth could explain the massive accumulation of the precursor.

Most of the radioactive proteins detected in minicells carrying pSC101-*ompA*+ and exhibiting smaller apparent molecular weights than protein II* are likely to be degradation products of this protein. The sum of their molecular weights is approximately 250,000, which is still just within the coding capacity of the cloned DNA fragment. It appears most unlikely, however, that this fragment should code almost exclusively for envelope proteins or for proteins that are not soluble under the conditions of envelope isolation and that all of them should be smaller than protein II*. Furthermore, the four polypeptides with the largest apparent molecular weights are also precipitated by anti-II* immunoglobulin.

It may be that the minicell envelope becomes overloaded with protein II* and its precursor and can no longer incorporate, quantitatively or correctly, the latter, which leads to degradation of newly made precursor. In agreement with this interpretation is the observation that radioactivity in the accumulated putative precursor can be only partially chased with cold methionine in the presence of chloramphenicol (data not shown), indicating that processing is blocked and thus opening the way to degradation processes.

The gene for a conditional major outer membrane protein of unknown function, polypeptide *a* [not produced as a major protein at growth temperatures of 32°C or below (54, 55)], has recently been accidentally cloned in pSC101 (45). It is of interest to note that the synthesis of this protein in minicells harboring the relevant plasmid (pMC 44) did not cause accumulation of any other outer membrane-associated protein. Thus, this protein may not be synthesized in precursor form, as also appears to be the case with several outer or plasma membrane-associated *tra* gene products [DNA transfer genes of the *E. coli* K-12 sex factor (56)]. Alternatively, and perhaps more likely because protein *a* is not normally produced in such large quantities as protein II*, the former's synthesis in minicells may not lead to such massive accumulation as has been shown here for protein II* and its (still putative) precursor. In line with this view, neither indications for degradation of protein *a* produced in minicells nor an influence on other major outer membrane proteins in cells carrying pMC44 have been observed by Gayda and Markovitz (45).

We are grateful to Drs. John Reeve and Mark Achtman for very helpful advice regarding preparation and handling of minicells.

1. Garten, W., Hindennach, I. & Henning, U. (1975) *Eur. J. Biochem.* **59**, 215–221.
2. DiRienzo, J. M., Nakamura, K., & Inouye, M. (1978) *Annu. Rev. Biochem.* **47**, 481–532.
3. Endermann, R., Krämer, C. & Henning, U. (1978) *FEBS Lett.* **86**, 21–24.
4. Van Alphen, L., Havekes, L., & Lugtenberg, B. (1977) *FEBS Lett.* **75**, 285–290.
5. Datta, D. B., Arden, B. & Henning, U. (1977) *J. Bacteriol.* **131**, 821–829.
6. Skurray, R. A., Hancock, R. E. W. & Reeves, P. (1974) *J. Bacteriol.* **119**, 726–735.
7. Schweizer, M. & Henning, U. (1977) *J. Bacteriol.* **129**, 1651–1652.
8. Manning, P. A., Pugsley, A. P. & Reeves, P. (1977) *J. Mol. Biol.* **116**, 285–300.
9. Sonntag, I., Schwarz, H., Hirota, Y. & Henning, U. (1978) *J. Bacteriol.* **136**, 280–285.
10. Sekizawa, J., Inouye, S., Halegoua, S. & Inouye, M. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1126–1133.
11. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
12. Inouye, S., Wang, S., Sekizawa, J., Halegoua, S. & Inouye, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1004–1008.
13. De Leij, L., Kingma, J. & Witholt, B. (1978) *Biochim. Biophys. Acta* **512**, 365–376.
14. Manning, P. A., Puspurs, A. & Reeves, P. (1976) *J. Bacteriol.* **127**, 1080–1084.
15. Datta, D. B., Krämer, C. & Henning, U. (1976) *J. Bacteriol.* **128**, 834–841.
16. Erlich, H. A., Cohen, S. N. & McDevitt, H. O. (1978) *Cell* **13**, 681–689.
17. Broome, S. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2746–2749.
18. Henning, U., Sonntag, I. & Hindennach, I. (1978) *Eur. J. Biochem.* **92**, 491–498.
19. Levy, S. B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2900–2904.
20. Garten, W. & Henning, U. (1976) *FEBS Lett.* **67**, 303–305.
21. Low, K. B. (1972) *Bacteriol. Rev.* **36**, 587–607.
22. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 433.
23. Reeve, J. N. (1977) *J. Bacteriol.* **131**, 363–365.
24. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97–106.
25. Mertens, G. & Reeve, J. N. (1977) *J. Bacteriol.* **129**, 1198–1207.
26. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
27. Sharp, P. A., Hsu, M. J., Ohtsubo, E. & Davidson, N. (1972) *J. Mol. Biol.* **71**, 471–497.
28. Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G. & Overath, P. (1978) *Mol. Gen. Genet.* **159**, 239–248.
29. Philippsen, P., Lee, C. S. & Davis, R. W. (1977) in *Molecular Approaches to Eukaryotic Genetic Systems*, eds. Wilcox, G. & Abelson, J. (Academic, New York), pp. 63–72.
30. Cameron, J. R., Panasenko, S. M., Lehman, I. R. & Davis, R. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3416–3420.
31. Cohen, S. N., Chang, A. C. Y., Boyer, H. W. & Helling, R. B. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3240–3244.
32. Cohen, S. N., Chang, A. C. Y. & Hsu, L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110–2114.
33. Meagher, R. B., Tait, R. C., Betlach, M. & Boyer, H. W. (1977) *Cell* **10**, 521–536.
34. Chai, T. & Foulds, J. (1974) *J. Mol. Biol.* **85**, 465–474.
35. Henning, U., Schwarz, H. & Chen, R. (1979) *Anal. Biochem.*, in press.

36. Raetz, C. R. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2274–2278.
37. Clarke, L. & Carbon, J. (1976) *Cell* **9,** 91–99.
38. Nishimura, Y., Takeda, Y., Nishimura, A., Suzuki, H., Inouye, M. & Hirota, Y. (1977) *Plasmid* **1,** 67–74.
39. Braun, V. (1975) *Biochim. Biophys. Acta* **415,** 335–377.
40. Bolivar, F. (1978) *Gene* **4,** 121–136.
41. Thomas, M., Cameron, J. R. & Davis, R. W. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4579–4583.
42. Gottesman, M. E. & Yarmolinsky, M. B. (1968) *J. Mol. Biol.* **31,** 487–505.
43. Struhl, K., Cameron, J. R. & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. USA.* **73,** 1471–1475.
44. Manning, P. A. & Reeves, P. (1976) *Biochem. Biophys. Res. Commun.* **71,** 466–471.
45. Gayda, R. C. & Markovitz, A. (1978) *J. Bacteriol.* **136,** 369–380.
46. Tait, R. C., & Boyer, H. W. (1978) *Cell* **13,** 73–81.
47. Thomas, M. & Davis, R. W. (1975) *J. Mol. Biol.* **91,** 315–328.
48. Halegoua, S., Hirashima, A. & Inouye, M. (1974) *J. Bacteriol.* **120,** 1204–1208.
49. Berg, P. (1977) in *Miami Winter Symposia*, eds. Scott, W. A. & Werner, R. (Academic, New York), Vol. 13, pp. 1–34.
50. Rao Movva, N., Katz, E., Asdourian, P. L., Hirota, Y. & Inouye, M. (1978) *J. Bacteriol.* **133,** 81–84.
51. Ryter, A., Shuman, H. & Schwartz, M. (1975) *J. Bacteriol.* **122,** 295–301.
52. Smit, J. & Nikaido, H. (1978) *J. Bacteriol.* **135,** 687–702.
53. Lee, N. & Inouye, M. (1974) *FEBS. Lett.* **39,** 167–170.
54. Lugtenberg, B., Peters, R., Bernheimer, H. & Berendser, W. (1976) *Mol. Gen. Genet.* **147,** 251–262.
55. Bassford, P. J., Diedrich, D. L., Schnaitman, C. L. & Reeves, P. (1977) *J. Bacteriol.* **131,** 608–622.
56. Kennedy, N., Beutin, L., Achtman, M., Skurray, R., Rahmsdorf, U. & Herrlich, P. (1977) *Nature (London)* **270,** 580–585.